Neutrophil oxidative burst activates ATM to regulate cytokine production and apoptosis

CJ Harbort[1], Paulo Vitor Soeiro-Pereira[2], Horst von Bernuth[3-4], Angela M. Kaindl[5-7], Beatriz Tavares Costa-Carvalho[8], Antonio Condino-Neto[9], Janine Reichenbach[10-13], Joachim Roesler[14], Arturo Zychlinsky[1]* and Borko Amulic[1]*.

Affiliations
[1] Department of Cellular Microbiology, Max Planck Institute for Infection Biology, Berlin, Germany;
[2] Department of Pathology, Federal University of Maranhão, Brazil;
[3] Department of Pediatric Pneumology and Immunology, Outpatient Clinic for Primary Immunodeficiencies, Charité Medical School, Berlin, Germany;
[4] Labor Berlin, Section for Immunology, Charité – Vivantes GmbH, Berlin Germany;
[5] Institute of Cell Biology and Neurobiology, Charité – Universitätsmedizin Berlin, Germany;
[6] Department of Pediatric Neurology, Charité – Universitätsmedizin Berlin, Germany;
[7] Sozialpädiatrisches Zentrum (SPZ), Charité – Universitätsmedizin Berlin, Germany
[8] Department of Pediatrics, Federal University of São Paulo Medical School, São Paulo, Brazil;
[9] Department of Immunology, Institute of Biomedical Sciences - University of São Paulo, Brazil;
[10] Division of Immunology, University Children's Hospital, and Children's Research Centre, University of Zürich, Switzerland;
[11] Swiss Center for Regenerative Medicine (SCRM), University of Zürich, Switzerland;
[12] Center for Applied Biotechnology and Molecular Medicine (CABMM), University of Zürich, Switzerland;
[13] Zurich Centre for Integrative Human Physiology (ZIHP), University of Zürich, Switzerland;
[14]Department of Pediatrics, University Hospital TU Dresden, Dresden, Germany

Corresponding author:

Borko Amulić, Charitéplatz 1, 10117 Berlin, Germany
amulic@mpiib-berlin.mpg.de
Phone: +493028460358 Fax: +493028460301

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*A.Z. and B.A. contributed equally to this work.

Key points

- Activation of ATM kinase modulates neutrophil functions and is dependent on the oxidative burst.
- Neutrophils from ataxia telangiectasia patients overproduce inflammatory cytokines and have a prolonged lifespan

Abstract

Neutrophils play an essential role in the initial stages of inflammation by balancing pro- and anti-inflammatory signals. Among these signals are the production of pro-inflammatory cytokines and the timely initiation of anti-inflammatory cell death via constitutive apoptosis. Here we identify ataxia telangiectasia mutated (ATM) kinase as a modulator of these neutrophil functions. Ataxia-telangiectasia (AT) is a pleiotropic multisystem disorder caused by mutations in the gene encoding ATM, a master regulator of the DNA damage response. In addition to progressive neurodegeneration and high rates of cancer, AT patients suffer from numerous symptoms that can be linked to chronic inflammation. We report that neutrophils isolated from patients with AT overproduce pro-inflammatory cytokines and have a prolonged lifespan compared to healthy controls. This effect is partly mediated by increases in activation of p38 MAP kinase. Furthermore, we show that the oxidative burst, catalyzed by NADPH oxidase, can activate ATM in neutrophils. Finally, activation of ATM and DNA damage signaling suppress cytokine production and can abrogate the overproduction of IL-8 in ROS-deficient cells. This reveals a novel mechanism for the regulation of cytokine production and apoptosis, establishing DNA damage as a downstream mediator of immune regulation by reactive oxygen species. We propose that deficiencies in the DNA damage response, like deficiencies in the oxidative burst seen in chronic granulomatous disease, could lead to pathological inflammation.

Introduction

The inflammatory response is tightly regulated to control its duration and magnitude and to limit the extent of collateral damage to host tissues. Improper regulation can lead to exaggerated inflammatory responses and pathology. Chronic inflammation is destructive to tissues and is a pathological component of many diverse diseases including cancer, cardiovascular disease, insulin-resistant diabetes as well as neurological diseases.

Ataxia-telangiectasia (AT) is a pleiotropic, recessive genetic disorder that occurs in an estimated one in 40,000 to 100,000 live births. AT affects multiple organ systems and is characterized by progressive neurodegeneration, radiosensitivity, sterility, growth defects, variable immunodeficiency and increased susceptibility to cancer[1]. Until now, immune dysfunction in AT has been characterized by improper B- and T-cell maturation[2-5], immunoglobulin deficiencies and impaired antibody responses observed in about two-thirds of patients[6,7]. Patients often suffer from recurring respiratory tract infections and chronic interstitial inflammatory lung disease, though opportunistic infections are uncommon[8,9]. Many symptoms in AT patients, including increased incidence of autoimmunity[10], oxidative stress[11], cardiovascular disease, insulin resistance, and elevated serum levels of inflammatory cytokines[12,13], suggest that they also suffer from pathological inflammation. Furthermore, there are several reports on patients presenting with chronic cutaneous granulomas and pulmonary inflammation without apparent underlying infections[14-17]. Although the immune defects in the adaptive arm of the immune system associated with this disease have been well studied, the effects on the innate immune system remain poorly understood.

Biallelic mutations in *ataxia-telangiectasia mutated* (*ATM*) are the underlying cause of AT. ATM is a large serine/threonine protein kinase involved in various cellular processes[18]. ATM is an important sensor of DNA damage, along with ATR (ataxia telangiectasia and RAD3-related) and DNA-PK (DNA-dependent protein kinase). In response to genotoxic insults, ATM is phosphorylated and then initiates and coordinates the DNA damage response. DNA damage signaling arrests the cell cycle until the lesions are resolved or, if the damage is beyond repair, can initiate apoptosis. ATM and

DNA-PK are activated in response to double strand breaks, and ATR by single strand breaks and incompletely replicated DNA[19]. Substrates of these kinases include histone variant H2A.X (a marker of DNA damage known as γ-H2A.X when phosphorylated), cell cycle regulators Chk1 and Chk2, as well as the apoptosis regulator p53[18]. The role of ATM in regulating cellular processes is not limited to DNA damage; it also coordinates responses to other forms of stress to restore homeostasis[20]. Notably, ATM was recently shown to be activated by oxidative stress[21] and was suggested to be involved in redox regulation.[22]

Neutrophils are the first cells recruited in large numbers to sites of inflammation where, in addition to antimicrobial activity, they exert both pro- and anti-inflammatory functions to balance the immune response[23]. Recognition of microbial components via pattern recognition receptors stimulates neutrophils by activating NF-κB and initiating mitogen-activated protein kinase (MAPK) signaling cascades. These pathways result in the production and secretion of pro-inflammatory cytokines, including interleukin 8 (IL-8) and macrophage inflammatory protein-1α (MIP-1α), which recruit and activate more immune cells. Activated neutrophils additionally mount an oxidative burst, producing large amounts of reactive oxygen species (ROS) via the NADPH oxidase complex. ROS are highly volatile molecules that damage many cellular components of microbes as well as the host, and additionally function as signaling molecules[24].

Neutrophils contribute to the regulation of inflammation through multiple processes. Their lifespan is tightly regulated to limit their pro-inflammatory functions. Additionally, apoptosis of the large population of infiltrating neutrophils provides a strong anti-inflammatory signal via efferocytosis, the removal of apoptotic cells via phagocytosis by other immune cells. Efferocytosis dampens pro-inflammatory cytokine production and initiates the resolution of inflammation[25,26]. In addition to antimicrobial activity, the oxidative burst in phagocytes contributes to regulation of both cytokine production and apoptosis, thus playing an important role in balancing inflammation[27]. Although the exact mechanisms are still not known, deficiencies in the neutrophil oxidative burst result in chronic granulomatous disease, characterized by impaired host defenses and hyperinflammation.

Here we show that neutrophils from AT patients overproduce pro-inflammatory cytokines and have a longer lifespan than those from healthy controls. We demonstrate that the activation of ATM and the DNA damage response in neutrophils normally plays a role in regulating cytokine production and apoptosis in a manner dependent on NADPH oxidase activity. Furthermore, we describe a similar effect of ATM inhibition on monocyte cytokine production, suggesting the existence of a common regulatory mechanism in myeloid cells. The absence of ATM activity, as with the oxidative burst, could thus disrupt immune regulation and potentially tip the scales in the direction of chronic inflammation.

Methods

Donor consent

Our study was conducted in accordance with the Helsinki Declaration. Blood samples were collected with approval from the ethical committees of each institution. Informed consent was provided by all patients, or by their parents, in the case of children. At the time of blood donation participants displayed no signs of infection or malignancy. Patients were receiving no treatments or medications apart from regular intravenous immunoglobulin therapy.

Neutrophil and Monocyte Isolation

Human neutrophils were isolated by centrifuging heparinized venous blood over Histopaque 1119 (Sigma-Aldrich) and subsequently over a discontinuous Percoll (Amersham Biosciences) gradient as previously described[28]. Peripheral blood monocytes were isolated by MACS selection using magnetically-labeled CD14 microbeads (Miltenyi Biotec). Cell preparations were at least 95% neutrophils or monocytes. were cultured in RPMI (Gibco) supplemented with 100 units/ml penicillin and streptomycin, 292 µg/ml L-glutamine (Gibco) and 10% fetal calf serum (Sigma).

Cytokine Production Assays

Cells were seeded in 96-well plates with indicated inhibitors or DMSO control and stimulated 18 hours with 100ng/ml lipopolysaccharide (LPS from *Salmonella typhimurium* (TLR grade), (Enzo Life Sciences), 10µg/ml opsonized Zymosan (Sigma-Aldrich), MOI 100 heat killed *Listeria monocytogenes* (HKLM, InvivoGen), or 1µg/ml flagellin (from *S. typhimurium*, InvivoGen). Zymosan was opsonized with human plasma from multiple donors. Inhibitors of ATM (KU-55933 and KU-60019, 10µM unless otherwise stated), ATR (VE-821, 1µM), DNA-PK (NU-7441, 1µM), Chk2 (PV-1019, 10µM), and p53 (Pifithrin-µ, 1µM) were obtained from Calbiochem. Cisplatin and etoposide were from Calbiochem and Sigma-Aldrich, respectively. P38 inhibitor (SB203580, 5 µM) and ERK inhibitor (PD98059, 5 µM) were from New England Biolabs. Cytokine concentrations were measured by enzyme-linked immunosorbent assay according to manufacturer's instructions (R&D systems DuoSet ELISAs).

Annexin V Viability Assay

Neutrophil viability was determined using the PE-Annexin V apoptosis kit (BD Biosciences). Briefly, neutrophils were resuspended at the indicated time points, washed in Annexin V binding buffer, stained with PE-Annexin V and vital dye propidium iodide (PI) and then analyzed by flow cytometry. Annexin V/PI double negative neutrophils were defined as viable (Supplemental Figure S3). **We found that we typically recover more than 90% of the cells after 18 hours.** At least 10,000 cells were measured per sample.

Quantitative Real Time PCR

RNA was isolated with RNeasy mini kit (Qiagen). cDNA was made using High-capacity RNA-to-cDNA kit (Applied Biosystems) according to manufacturer's protocol. Quantative Real-time PCR was performed on StepOnePlus Real-Time PCR System with 2x Fast SYBR Green master mix (Applied Biosystems) according to protocol with cDNA made from 10ng of RNA per reaction. Previously verified primers[29] were used for IL-8 F 5'-CTGGCCGTGGCTCTCTTG-3' and R 5'-CCTTGGCAAAACTGCACCTT-3', MIP-1α F 5'-AGCTGACTACTTTGAGACGAGCA-3' and R 5'-CGGCTTCGCTTGGTTAGGA-3', and

housekeeping gene β2-microglobulin F 5'-CTCCGTGGCCTTAGCTGTG-3' and R 5'-TTTGGAGTACGCTGGATAGCCT-3'. Data were analyzed using StepOne software and expressed as relative amount of IL-8 or MIP-α product as determined from pooled standard curve divided by relative amount of β2- microglobulin product at each time point.

Intracellular IL-8 staining

Neutrophils were incubated with 3 µg/ml Brefeldin A and stimulated with LPS as described above. At indicated time points, neutrophils were resuspended and fixed with 2% PFA, washed and resuspended in PBS 1% BSA and stored at 4°C. Cells were permeabilized with saponin, washed, and stained with PE-mouse anti-human IL-8 or isotype control antibody from BD Pharminogen, and analyzed by flow cytometry on a MACSQuant. At least 10,000 cells were measured per sample.

Western Blot

Lysates were made from $2x10^6$ cells resuspended in 100 µl of Laemmli's buffer supplemented with protease inhibitor cocktail (Sigma), Halt! Phosphatase inhibitor (Fischer scientific) and 2µM neutrophil elastase inhibitor V (Calbiochem). Protein lysates were resolved by SDS-PAGE followed by analysis by western immunoblotting. Anti-phospho-histone H2A.X (Ser139) was from Millipore. All other antibodies used were obtained from Cell Signaling Technologies.

Immunofluorescence

Neutrophils stimulated with 100nM phorbol 12-myristate 13-acetate (PMA) on glass coverslips were fixed with 2% paraformaldehyde 90 minutes after activation, permeabilized 5 minutes in ice cold acetone, then blocked at 37˚C for 1 hour in 1% bovine serum albumin, 5% normal donkey serum and 15% cold water fish gelatin (Sigma-Aldrich). Samples were stained in blocking buffer with 2 µg/ml mouse anti-human phospho-ATM (Ser 1981) (Thermo scientific), followed by secondary antibody goat-anti mouse IgG conjugated to Alexa Fluor 568 (Invitrogen). Coverslips were mounted on ProLong Gold antifade mountant with DNA dye DAPI (Thermo Fisher Scientific). Images were taken with a Leica SP8 confocal microscope (Leica).

Results

We assayed the effects of ATM activity on neutrophil functions by incubating cells with various inflammatory stimuli in the presence of KU-55933, a highly specific small molecule inhibitor of ATM[30]. Neutrophils express functional ATM, which is phosphorylated in response to irradiation-induced DNA damage and this activation is abrogated in the presence of KU-55933 (Figure 1A). We found that this inhibitor caused a strong dose-dependent increase in the secretion of IL-8 and MIP-1α in response to LPS, a component of bacterial outer membranes (Figure 1B). The specificity of this effect was confirmed using a second inhibitor of ATM, KU-60019 (Supplemental Figure S1). We observed an increase in cytokine production in the presence of KU-55933 not only in response to LPS-stimulation, but also in response to opsonized zymosan, flagellin, and heat-killed *Listeria monocytogenes* (Figure 1C). An increase in the production of IL-6 in response to LPS and heat-killed *Listeria* was similarly observed (Supplemental Figure S2). Moreover, ATM inhibition in peripheral blood monocytes significantly increased the production of IL-1β, IL-6, and TNF in response to LPS stimulation (Figure 1D).

To determine if genetic deficiencies in ATM affect neutrophil cytokine production in patients, we measured IL-8 production from primary peripheral blood neutrophils isolated from 11 different patients with AT (mean age=16 years). One of these patients participated twice on separate occasions (n=12). All patients were free of infections and malignancy at the time of blood donation. AT diagnoses were confirmed by *ATM* gene sequencing (Supplemental Table S1). Neutrophils isolated from 12 sex-matched healthy donors were used as controls (mean age=28, n=12). Neutrophils were isolated and experiments performed with one AT patient and one control concurrently. Neutrophils from patients with AT produced significantly more IL-8 (mean = 1.73 ng/10^6 cells) in response to LPS than healthy controls (mean = 0.69 ng/10^6 cells) (Figure 2A). These data, complemented by our experiments with ATM inhibitors, show that cytokine production is increased in the absence of ATM activity.

Because neutrophil apoptosis is regulated by p53, which can be induced by ATM activation[31], we asked whether ATM deficiency affects neutrophil lifespan. Cell viability

was assayed by flow cytometry over an 18-hour time course by staining with PE-labeled annexin V (which binds exposed phosphatidylserine on the surface of apoptotic cells), and propidium iodide (PI, a vital DNA dye), at the indicated time points. We found that neutrophils from AT patients lived significantly longer than controls (Figure 2B). We confirmed this result by measuring viability with the Sub-G1 assay (Supplemental Figure S4). Taken together, these data show that primary neutrophils from AT patients overproduce inflammatory cytokines and delay constitutive apoptosis.

In response to LPS stimulation, ~~the~~ MAP kinases ~~ERK and p38~~ are activated by phosphorylation while IκBα, an inhibitor of the transcription factor NF-κB, is degraded. To determine if ATM regulates the activation or duration of these signaling pathways, we measured their activation in response to LPS stimulation by western blot. In a 3-hour time course of LPS stimulation we observed phosphorylation of ATM and histone H2A.X. Phosphorylation of ATM, but not H2A.X, in response to LPS was abrogated by the addition of KU-55933, while p38 phosphorylation was increased (Figure 3A and B). We ~~similarly~~ observed increased ERK phosphorylation and degradation of IκBα**, but only** at later timepoints **(Supplemental Figure S5).** We then used inhibitors of p38 and ERK to determine if IL-8 overproduction in the absence of ATM activity is dependent on these MAP kinases. P38 inhibition blocked IL-8 production in controls, and abrogated the overpoduction of IL-8 in response to ATM inhibition (Figure 3C).

Increased MAPK ~~and NF-κB~~ activation in the absence of ATM activity led to increased cytokine transcription. We observed an increase in both IL-8 and MIP-1α transcripts in the presence of KU-55933 compared to the housekeeping gene β_2-microglobulin by qPCR, (Figure 3D). Correspondingly, we observed an increase in IL-8 protein in neutrophils stimulated in the presence of KU-55933, as shown by intracellular antibody staining and analysis by flow cytometry (Figure 3E).

We next used a panel of small molecule inhibitors targeting specific kinases involved in the DNA damage response to determine if other members of this pathway influence cytokine production. Inhibition of ATR, DNA-PK and Chk2 increased IL-8 production in response to LPS, similar to ATM inhibition and consistent with their partially redundant roles in the DNA damage response (Figure 4A). In line with this, ATM and ATR inhibition synergistically increases cytokine production above the level of either

inhibitor alone (Supplemental Figure S5). Interestingly, inhibiting p53 drastically prolonged neutrophil life span (Figure 4B) but did not affect IL-8 production (Figure 4A). Thus, increased cytokine production in the absence of DNA damage signaling is not strictly linked to prolonged lifespan.

These data suggest that DNA damage signaling downregulates neutrophil cytokine production. To directly test this hypothesis, we pre-incubated neutrophils for one hour with cisplatin or etoposide, two chemical inducers of DNA damage, or exposed them to 10 Gy of γ-irradiation before stimulating them with LPS. These treatments induced DNA damage and activated ATM, as shown by western blot analysis of histone γ-H2A.X and phospho-ATM (Figure 4C), and significantly reduced the production of IL-8 (Figure 4D). Interestingly, treatment with etoposide or irradiation strongly activated ATM and reduced cytokine production without large effects on cell viability after 8 hours (Figure 4E). Together, these results show that activation of the DNA damage response suppresses cytokine production and contributes to neutrophil apoptosis.

Since ROS can cause oxidative damage to DNA, we hypothesized that the oxidative burst mounted upon stimulation may activate the DNA damage response in neutrophils. We tested this by using PMA and opsonized zymosan, two potent inducers of the oxidative burst (Supplemental Figure S6). In response to PMA, we observed an accumulation of phospho-ATM staining in the nuclei of stimulated neutrophils (Supplemental Figure S7). We tested the ROS dependence of this activation using pyrocatechol (Figure 5A), a cell permeable ROS scavenger capable of neutralizing the oxidative burst as shown in Supplemental Figure S8. In the presence of pyrocatechol, activated neutrophils showed less phosphorylation of ATM compared to controls (Figure 5A). We confirmed the activation of DNA damage signaling by western blot analysis in neutrophils stimulated with PMA and opsonized zymosan (Figure 5B). Stimulation caused a strong induction of phospho-ATM, phospho-BRCA-1 (a substrate of ATM) and γ-H2A.X in control cells, but not in the presence of pyrocatechol. Phosphorylation of ATM and H2A.X in response to irradiation-induced DNA damage, however, was not affected by ROS scavenging.

To test if the oxidative burst is the endogenous source of ROS activating the DNA damage response, we similarly evaluated ATM activation in neutrophils from patients with chronic granulomatous disease (CGD). CGD is a genetic disorder in which

neutrophils fail to mount an oxidative burst due to deficiencies in NADPH oxidase (Supplemental Figure S9). Interestingly, we could show that ATM is effectively activated in neutrophils isolated from CGD patients in response to irradiation, but not in response to ROS-inducing stimuli (Figure 5C). This confirms that NADPH oxidase-derived ROS are required for activating the DNA damage response in stimulated neutrophils.

Our results predict that CGD neutrophils, similar to AT neutrophils, would produce more cytokines due to impaired activation of ATM and the DNA damage response. We confirmed this by comparing cytokine production from patients with the two deficiencies: neutrophils isolated concurrently from both a CGD and an AT patient produced more IL-8 than cells from a healthy control (Figure 5D). We obtained similar results when incubating neutrophils with ROS scavenger pyrocatechol or ATM inhibitor KU-55933 (Figure 5E). This is consistent with published reports on the hyperactivation of CGD phagocytes[32-34]. Furthermore, we could rescue the IL-8 overproduction phenotype of ROS-scavenged cells by exogenously inducing DNA damage (Figure 5F). Etoposide treatment potently decreased IL-8 production from pyrocatechol-treated neutrophils back to levels similar to control cells.

Discussion

Our study is, to the best of our knowledge, the first to examine primary innate immune cells from AT patients. We show that ROS-mediated activation of DNA damage signaling in neutrophils suppresses the production of pro-inflammatory cytokines and activates apoptosis. Accordingly, we report that deficiencies in the DNA damage response, similar to deficiencies in the oxidative burst, result in the overproduction of cytokines and delayed apoptosis. We demonstrate a key role for ATM and show that cytokine production and apoptosis are dysregulated in neutrophils from patients with AT. These results further our understanding of the ROS-mediated regulation of inflammation and indicate an interesting link between two diverse diseases, AT and CGD. Moreover, our results suggest that innate immune dysfunction may drive inflammation in AT.

The specific mechanisms by which ATM and the DNA damage response contribute to the regulation of cytokine production are not completely clear. The effect is at least partially mediated by modulating well-described MAPK signaling pathways. Our

data demonstrates an important role for p38 and suggests one for ERK as well. It has previously been reported that ATM and other members of the DNA damage response can affect the activation of MAP kinases and NF-κB[35,36], which are important regulators of cell survival and inflammation[37].

Consistent with our findings, several lines of evidence have recently emerged which point to ATM having a major role in suppressing inflammation. In human dendritic cells ATM was shown to be a negative regulator of IL-23 production[38]. Furthermore, pharmacological induction of the DNA damage response reduces levels of pro-inflammatory cytokines and increases survival in septic mice[39]. This protective effect was shown to be partly mediated by ATM activation, although the researchers conclude that this is a lung specific mechanism not mediated by blood cells. Similarly, Härtlova et al[40] reported that ATM deficiency in macrophages increases production of type I interferon. The authors, however, do not suggest a direct role for ATM in interferon regulation. Rather, they propose that unrepaired DNA lesions in AT cells activate Stimulator of IFN Genes (STING) and prime the type I interferon system. This mechanism and the ROS-mediated one proposed by our study are not mutually exclusive. It would be interesting to examine if other cytokines, including IL-8, are also upregulated in AT macrophages.

Our data indicate that ATM is not the sole member of the DNA damage response that regulates neutrophil function. Inhibition of DNA damage sensors ATR and DNA-PK, as well as Chk2, a substrate of ATM, also increased cytokine production. These sensors are often described as each recognizing a specific type of damage to DNA and executing parallel but discrete responses through different downstream mediators. In reality there is much overlap and crosstalk between these pathways and substrates, meaning they may be partially redundant[41]. Indeed, we observe that simultaneous inhibition of ATM and ATR synergistically increases cytokine production more than either inhibitor alone (Supplemental figure S5). Chk2 has been shown to affect MAP kinase activation[42,43] and can also be activated by ATR, though less efficiently than by ATM[44]. Thus, these kinases may converge on Chk2 to regulate cytokine production.

Neutrophil cytokine production and lifespan are closely linked and may affect each other. Secretion of IL-8 can increase lifespan by autocrine signaling[45], and is one possible mechanism causing increased viability in AT cells. We can, however, conclude

that the regulation of cytokine production by the DNA damage response can be uncoupled from its regulation of apoptosis. Inhibiting p53 dramatically prolonged neutrophil lifespan, but did not increase IL-8 production. Futhermore, normalizing cytokine production to viable cells at an earlier timepoint clarifies that when ATM is inhibited, the production of IL-8 increases in magnitude and is not only a result of an increased lifespan (Supplemental Figure 10). Similarly, inducing DNA damage by treatment with etoposide, or irradiation reduced cytokine production, but had no effect on apoptosis in the time span under investigation.

One of the most prominent neutrophil responses to activating stimuli, in addition to cytokine production, is the oxidative burst. Production of superoxide and its derivatives hydrogen peroxide and hypochlorous acid is thought to contribute to microbial killing, and also serve important signaling functions[23]. ROS are known to induce DNA damage and to activate ATM directly by oxidation[21], and were thus prime candidates for triggering ATM activity in activated neutrophils. By comparing neutrophil responses in AT and CGD patients, we identified the oxidative burst as an activator of ATM important for regulating cytokine production. A side-by-side comparison of IL-8 production in control, CGD and AT neutrophils reveals that ROS and ATM deficiencies phenocopy each other in this respect. Exogenous induction of DNA damage can rescue the cytokine overproduction phenotype of ROS-scavenged neutrophils and further supports this model. It is not yet clear whether the activation of ATM by ROS is directly mediated by oxidation of ATM, or via oxidative damage to the DNA. Phosphorylation of H2A.X in response to LPS when ATM is inhibited indicates that this activation is via DNA damage, and may be increased in ATM deficiency due to inefficient repair.

Our results thus suggest the following model for the initiation and resolution of inflammation by myeloid cells: 1) Neutrophil activation leads to activation of MAP kinases and initiation of oxidative burst and cytokine production. 2) NADPH oxidase-derived ROS, in addition to contributing to microbial killing, also activate ATM. 3) ATM signaling participates in initiating the resolution phase by limiting cytokine production and promoting anti-inflammatory neutrophil cell death. Thus, augmented cytokine production coupled with decreased apoptosis rates could result in a synergistic increase in the magnitude and lifespan of neutrophil inflammatory responses in AT patients.

Furthermore, our model suggests a potential mechanistic link between the pathologies observed in two completely different diseases: AT and CGD (Supplemental Figure S11). ROS are ubiquitous and promiscuous molecules, playing a role in signaling and regulation of many pathways. In neutrophils, the oxidative burst is known to regulate antimicrobial responses, apoptosis, autophagy, hypoxia and oxidative stress responses, chemotaxis, and NETosis[23]. CGD is the result of dysregulation of all of these responses, which causes immunodeficiency and hyperinflammation. Similarly, AT is the result of dysfunctions in many pathways involving ATM. We propose that the ROS-mediated activation of ATM is an important response which is dysregulated in AT that affects cytokine production; a dysregulation that is shared by patients with CGD. Though these diseases are very different, they may have an inflammatory common denominator.

Chronic inflammation, granuloma formation, and elevated risk of autoimmune disorders in CGD are attributed to increased cytokine production and delayed apoptosis of neutrophils and macrophages[34]. We observe similar dysregulation of neutrophil functions in AT patients and believe this may also drive chronic inflammation and disease in AT. In agreement with this, several authors have reported instances of nonresolving, sterile skin granulomas in AT patients. Furthermore, both AT and CGD patients have an elevated risk of developing autoimmune diseases and auto-antibodies[46-49]. Prolonged inflammatory responses could drive and exacerbate the inflammatory symptoms of AT and even contribute to neurodegeneration and high rates of cancer. Several reports have suggested excessive inflammatory responses in a mouse model of AT, resulting in increased susceptibility to colitis[50] as well as inflammatory tissue damage in the lung[51].

There is currently no treatment available for AT other than palliative care. Interestingly, Chessa et al.[52] found that treatment with the anti-inflammatory steroid betamethasone improved neurological symptoms for AT patients, though it remains to be seen if this effect was mediated by the immunosuppressive properties of the drug. Our work further strengthens the argument that targeting inflammation may be a promising clinical intervention for some of the symptoms of this devastating disease.

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Authorship

Contribution: C.J.H. and B.A. designed and performed the experiments and analyzed the data; C.J.H. made the figures and wrote the manuscript; P.V.S.P., H.B., A.M.K., B.T.C.C., A.C.N., J.Reichenbach and J. Roesler arranged for blood donation by AT and CGD patients, provided facilities for experiments, and contributed helpful comments to the manuscript; A.Z. and B.A. directed the study and supervised the writing of the manuscript.
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Correspondence: Borko Amulić, Max Planck Institute for Infection Biology, Chariteplatz 1, 10117 Berlin, Germany; e-mail: amulic@mpiib-berlin.mpg.de.

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Figure legends:

Figure 1. Inhibition of ATM activity in neutrophils increases pro-inflammatory cytokine production. (A) Western blot of ATM, phosphorylated ATM, and γ-H2A.X in lysates from neutrophils exposed to gamma irradiation and incubated for 1 hour with and without 10μM ATM inhibitor KU-55933 (B) Neutrophil IL-8 (n=4) and MIP-1α (n=3) production in response to LPS stimulation in the presence of increasing KU-55933 concentration. Isolated peripheral blood neutrophils from healthy donors were incubated with ATM inhibitor and stimulated with LPS for 18 hours. Cytokine concentrations in supernatants were measured by ELISA. (C) Neutrophil IL-8 production in response to 18-hour stimulation with flagellin (1μg/ml), LPS (100ng/ml), opsonized zymosan (10μg/ml), or heat killed L. monocytogenes (HKLM, MOI = 100) in the presence of KU-55933 (10μM). (n=3-4). (D) Monocyte cytokine production in response to LPS stimulation in the presence of KU-55933. Isolated peripheral blood monocytes from healthy donors (n=10) were incubated with KU-55933 (10μM) and stimulated for 18 hours with LPS. Data in B-D are presented as the mean ± SEM of compiled experiments. Asterisks indicate significant increases: *P<0.05, **P<0.01, ***P<0.001 by paired t-test.

Figure 2. Primary neutrophils isolated from AT patients produce more IL-8 and have a prolonged lifespan. (A) IL-8 production from LPS-stimulated peripheral blood neutrophils isolated from AT patients (mean = 1.73 ng, n=12) or healthy controls (mean = 0.69 ng, n=12) 18 hours after stimulation. P = .0086, Mann-Whitney U Test. IL-8 concentrations in supernatants were measured by ELISA and normalized to cell number. Data are plotted as mean ± SEM. (B) 18-hour viability curve of primary neutrophils isolated concurrently from AT patients (n=5-7) and healthy controls (n=6-8). At the indicated timepoints, neutrophils were collected and stained with PE-annexin V and PI for viability. 10 000 cells per condition were analyzed by flow cytometry. Annexin V/PI-double negative cells were defined as viable. Data are plotted as mean ± SEM. Asterisks indicate significant increases: *P<0.05, **P<0.01, ***P<0.001 by paired t-test.

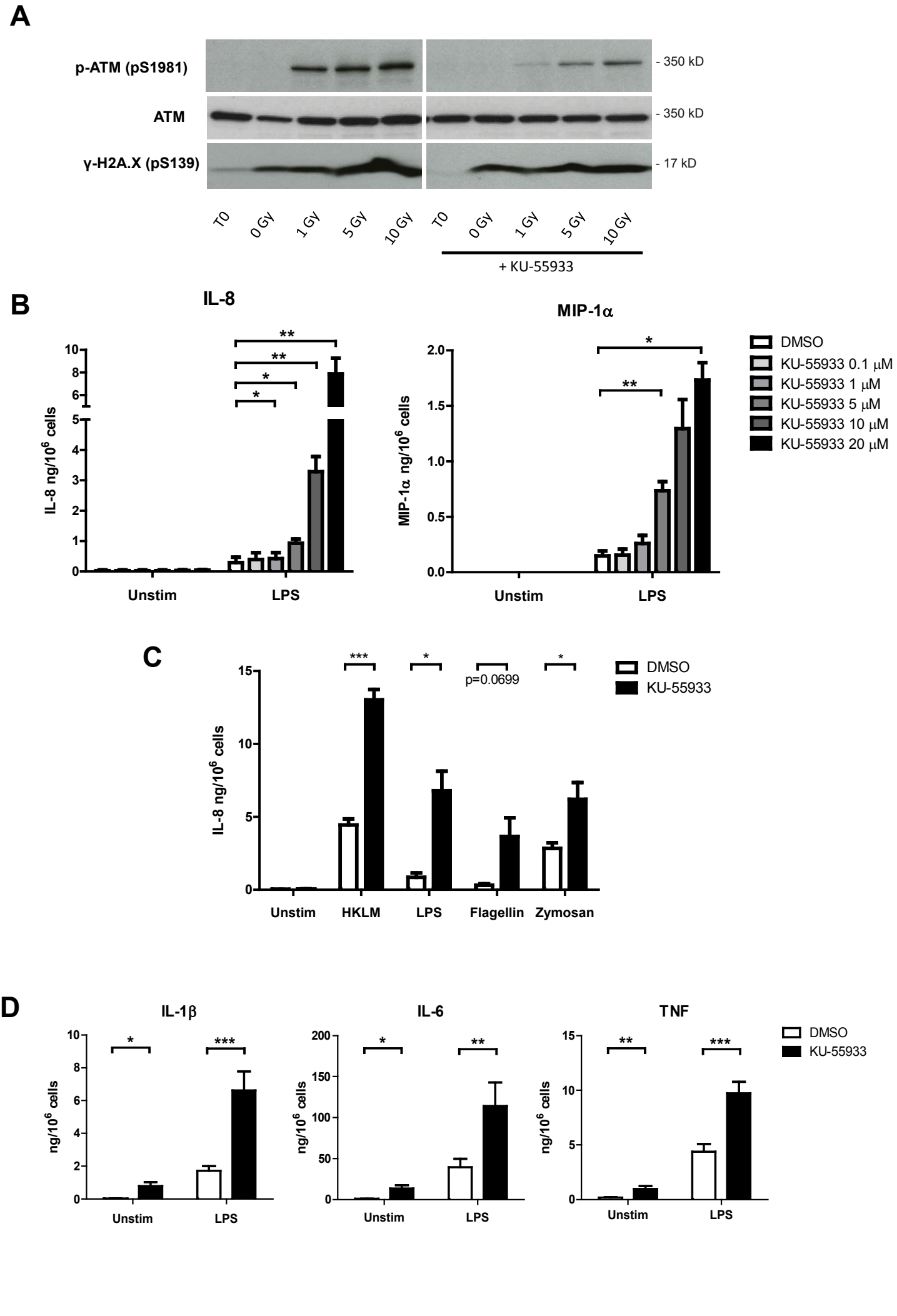
Figure 3. ATM inhibition increases ~~NF-κB and MAPK~~p38 activation as well as cytokine transcription and production in stimulated neutrophils. (A) Western blot analysis of phosphorylation of ATM, histone H2A.X, and ~~MAP kinases, and IκBα degradation~~p38 in neutrophil lysates over a 180-minute time course of LPS stimulation with and without 10μM KU-55933, representative blot of 4 experiments. (B) Relative levels of phospho-p38~~, phospho-ERK, and IκBα~~ quantified by densitometry in ImageJ. Data are expressed as signal relative to the loading control GAPDH (n=4). (C) Effect of p38 and ERK inhibitors on neutrophil IL-8 production in the presence of KU-55933 in response to LPS stimulation. IL-8 concentration in supernatants was measured after 18 hours stimulation (n=5) (D). Quantitative real-time PCR analysis of relative IL-8 and MIP-1α transcripts in cDNA from neutrophils stimulated with LPS with and without KU-55933. mRNA was isolated from LPS-stimulated neutrophils at the indicated timepoints, converted to cDNA, and analyzed by qPCR in triplicate (n=3). Data are expressed as the of relative amount of cytokine transcript divided by relative amount of housekeeping gene β2-microglobulin (n=3). Relative transcript amounts were calculated using a standard curve made from serial dilutions of a pooled sample. Data were analyzed using StepOnePlus software. (D) Intracellular IL-8 staining of LPS-stimulated neutrophils in the presence of KU-55933. Neutrophils were incubated with brefeldin A to block secretion and stimulated with LPS with or without ATM inhibitor. At the indicated time points, cells were fixed and stained with an anti-IL8 antibody and analyzed by flow cytometry. At least 10 000 cells were analyzed per sample. Grey fill represents unstimulated neutrophils, solid line LPS-stimulated DMSO control, and dashed line LPS-stimulated with 10μM KU-55933. Data in B-D are presented as the mean ± SEM of compiled experiments. Asterisks indicate significance: *P<0.05, **P<0.01, ***P<0.001 by paired t-test.

Figure 4. Activation of the DNA Damage Response downregulates neutrophil cytokine production. (A) Neutrophil IL-8 production in response to LPS stimulation in the presence of inhibitors of DNA damage response proteins. Neutrophils were incubated with the indicated inhibitors (inhibitor targets in parentheses) and stimulated 18 hours with LPS in triplicate. IL-8 concentrations in supernatants were measured by ELISA Data are plotted as the mean ± SEM of compiled experiments, (n=5). Black line

represents amount of IL-8 produced by LPS-stimulated control cells. (B) Neutrophil viability time course in the presence of p53 inhibitor pifithrin-μ (1μM) measured by PE-annexin V/PI staining, as before. (C) Western blot analysis of phosphorylation of ATM and histone H2A.X in neutrophil lysates made after 1 hour treatment with cisplatin, etoposide, and gamma-irradiation. (D) Neutrophil IL-8 production in response to 18-hour LPS stimulation after one hour exposure to the indicated DNA damage-inducing agents. Data are plotted as the mean ± SD. Results representative of 3 experiments. (E) Neutrophil viability after 1 hour incubation with indicated DNA damage-inducing treatment followed by 8 hour stimulation with LPS or left unstimulated. Viability was measured as described by PE-annexin V/PI staining. Asterisks indicate significance: **P*<0.05, ***P*<0.01, ****P*<0.001 by paired t-test.

Figure 5. Neutrophil oxidative burst activates the DNA damage response and regulates cytokine production. (A-D) NADPH oxidase-derived ROS activates the DNA damage response in stimulated neutrophils. (A) Phospho-ATM (red) and DNA (blue) staining of healthy neutrophils activated with PMA in the presence of 30μM ROS scavenger pyrocatechol. Neutrophils were stimulated for 90 minutes with PMA to induce ROS production, fixed, permeabilized, stained with anti-pATM (pS1981) antibody and visualized by confocal microscopy. (B-D) Western blot analysis of DNA damage response activation in lysates from neutrophils stimulated with PMA, opsonized zymosan, or 10 Gy irradiation in the absence or presence of pyrocatechol (B), or isolated from control and CGD patient (C). (D-E) IL-8 production from neutrophils deficient in ATM activity and ROS production. (D) IL-8 production from neutrophils isolated concurrently from a CGD patient, an AT patient, and a healthy control after 18 hour LPS stimulation (single experiment) Data are plotted as the mean ± SEM of triplicate stimulations. (E) IL-8 production from healthy control neutrophils incubated with ROS scavenger pyrocatechol or ATM inhibitor KU-55933, and stimulated 18 hours with LPS (n=3). IL-8 concentration in supernatants were measured by ELISA. (F) IL-8 production from neutrophils incubated with pyrocatechol, pre-treated for one hour with indicated concentrations of etoposide, and stimulated 18 hours with LPS (n=3). Data in E-F are presented as the mean ± SEM of compiled experiments. Asterisks indicate significance: **P*<0.05, ***P*<0.01, ****P*<0.001 by paired t-test.

Figure 1


A
p-ATM (pS1981)
ATM
γ-H2A.X (pS139)
- 350 kD
- 350 kD
- 17 kD
T0
0 Gy
1 Gy
5 Gy
10 Gy
T0
0 Gy
1 Gy
5 Gy
10 Gy
+ KU-55933
B
IL-8
MIP-1α
IL-8 ng/10^6 cells
MIP-1α ng/10^6 cells
Unstim
LPS
DMSO
KU-55933 0.1 μM
KU-55933 1 μM
KU-55933 5 μM
KU-55933 10 μM
KU-55933 20 μM
C
IL-8 ng/10^6 cells
p=0.0699
DMSO
KU-55933
Unstim
HKLM
LPS
Flagellin
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D
IL-1β
IL-6
TNF
ng/10^6 cells
Unstim
LPS
DMSO
KU-55933

Figure 2

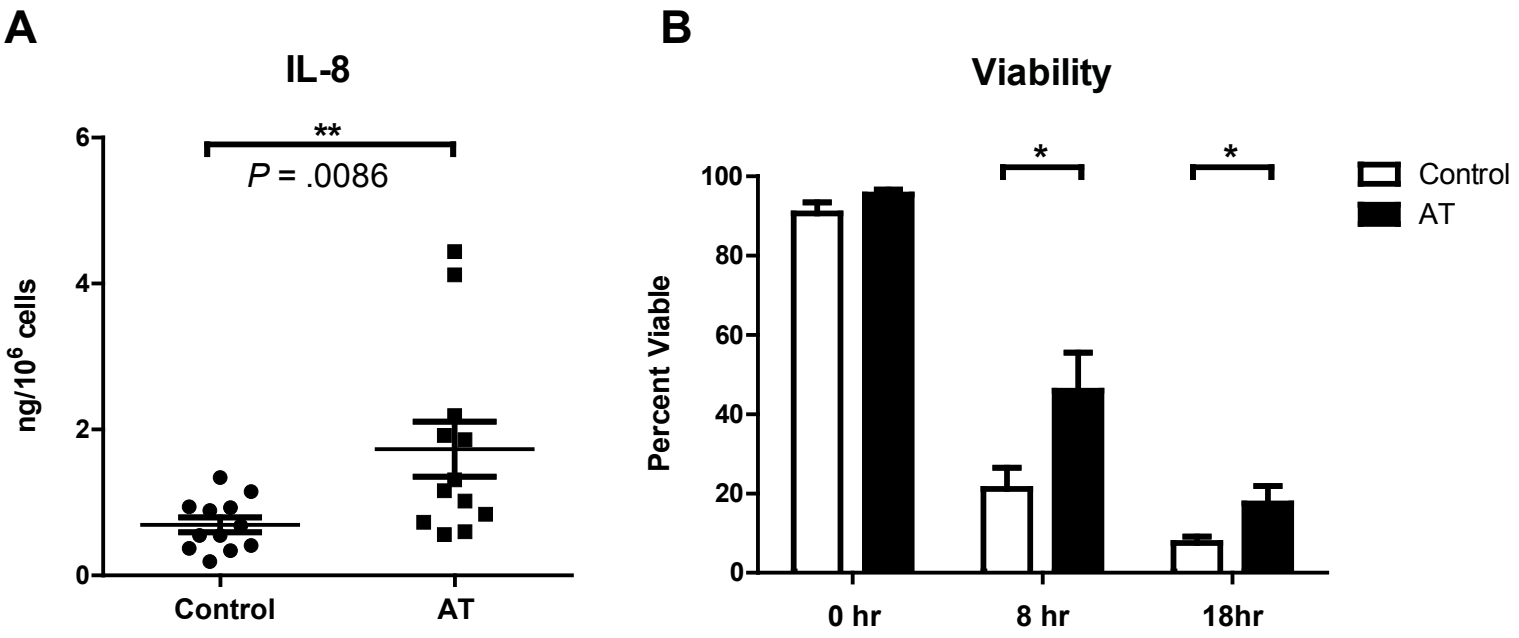

A
IL-8
**
P = .0086
ng/10^6 cells
6
4
2
0
Control
AT
B
Viability
*
*
Percent Viable
100
80
60
40
20
0
0 hr
8 hr
18hr
Control
AT

Figure 3

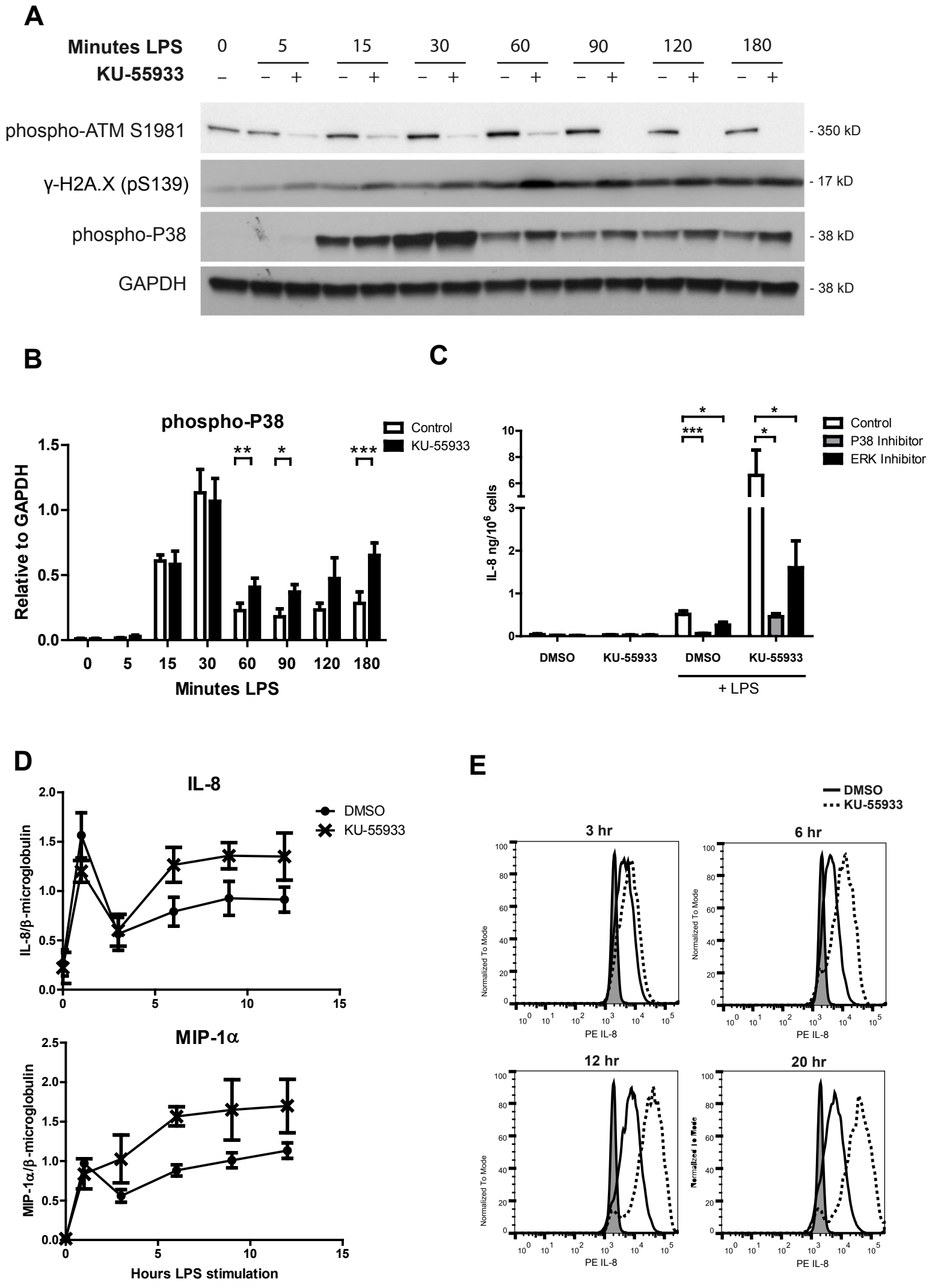

A
Minutes LPS
0
5
15
30
60
90
120
180
KU-55933
phospho-ATM S1981
γ-H2A.X (pS139)
phospho-P38
GAPDH
- 350 kD
- 17 kD
- 38 kD
- 38 kD
B
phospho-P38
Control
KU-55933
Relative to GAPDH
Minutes LPS
C
Control
P38 Inhibitor
ERK Inhibitor
IL-8 ng/10^6 cells
DMSO
KU-55933
DMSO
KU-55933
+ LPS
D
IL-8
DMSO
KU-55933
IL-8/β-microglobulin
MIP-1α
MIP-1α/β-microglobulin
Hours LPS stimulation
E
DMSO
KU-55933
3 hr
6 hr
12 hr
20 hr
Normalized To Mode
PE IL-8

Figure 4

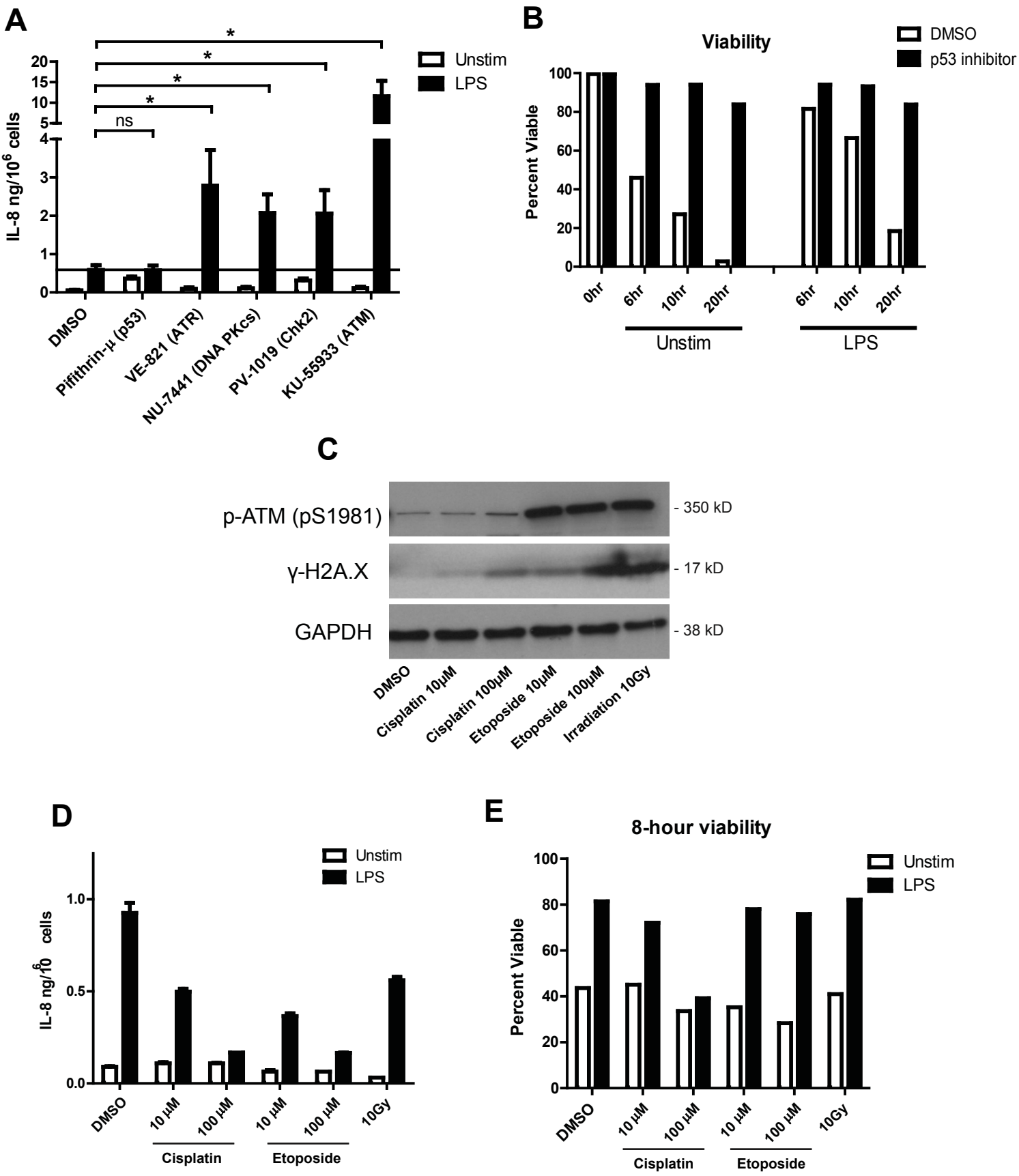
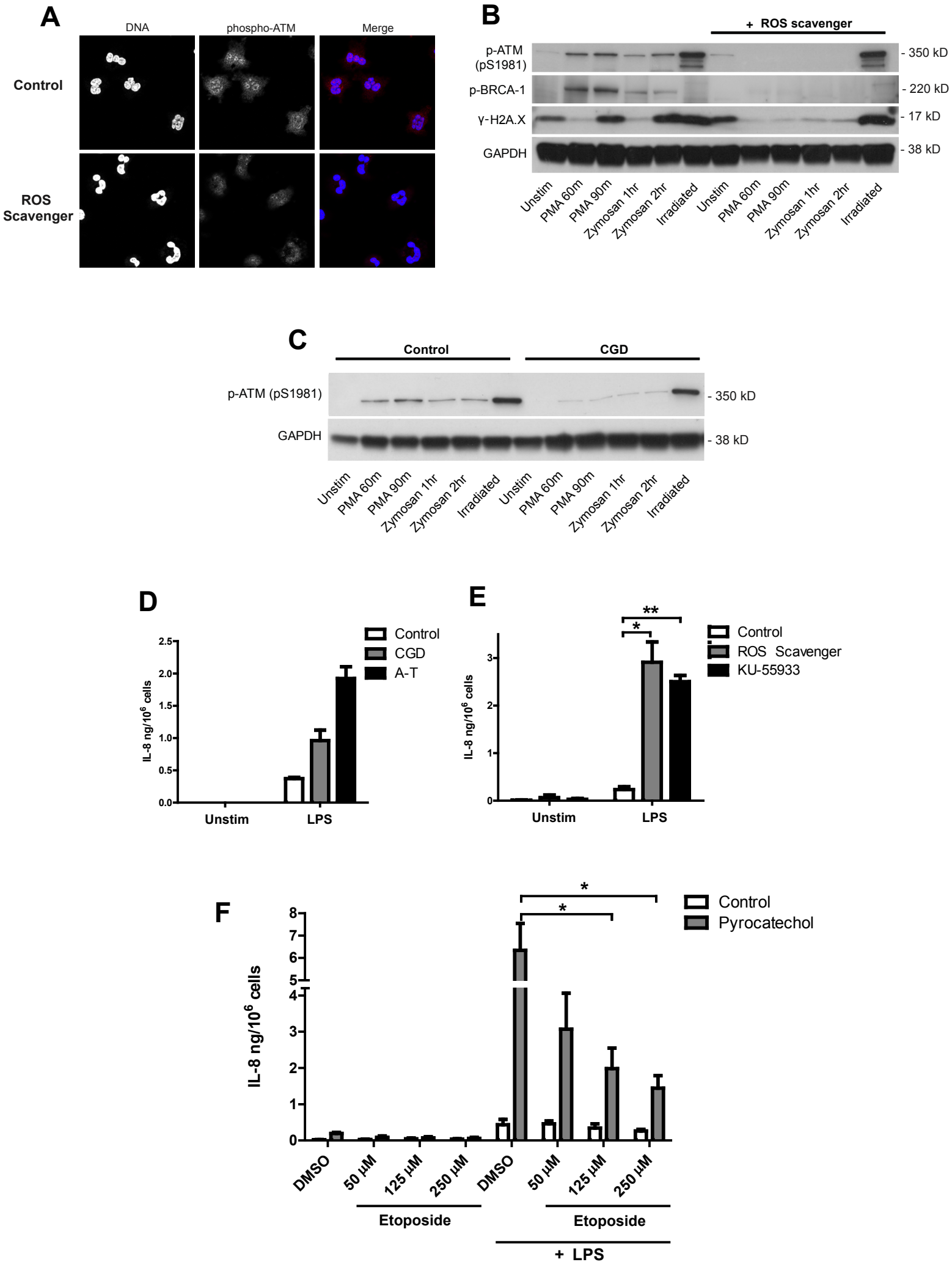

A
Unstim
LPS
IL-8 ng/10^6 cells
ns
*
DMSO
Pifithrin-μ (p53)
VE-821 (ATR)
NU-7441 (DNA PKcs)
PV-1019 (Chk2)
KU-55933 (ATM)
B
Viability
DMSO
p53 inhibitor
Percent Viable
0hr
6hr
10hr
20hr
Unstim
LPS
C
p-ATM (pS1981)
γ-H2A.X
GAPDH
- 350 kD
- 17 kD
- 38 kD
DMSO
Cisplatin 10μM
Cisplatin 100μM
Etoposide 10μM
Etoposide 100μM
Irradiation 10Gy
D
Unstim
LPS
IL-8 ng/10^6 cells
DMSO
10 μM
100 μM
Cisplatin
Etoposide
10Gy
E
8-hour viability
Unstim
LPS
Percent Viable
DMSO
10 μM
100 μM
Cisplatin
Etoposide

Figure 5


A
DNA
phospho-ATM
Merge
Control
ROS Scavenger
B
+ ROS scavenger
p-ATM (pS1981)
p-BRCA-1
γ-H2A.X
GAPDH
- 350 kD
- 220 kD
- 17 kD
- 38 kD
Unstim
PMA 60m
PMA 90m
Zymosan 1hr
Zymosan 2hr
Irradiated
C
Control
CGD
p-ATM (pS1981)
GAPDH
- 350 kD
- 38 kD
D
Control
CGD
A-T
IL-8 ng/10^6 cells
Unstim
LPS
E
Control
ROS Scavenger
KU-55933
IL-8 ng/10^6 cells
Unstim
LPS
F
Control
Pyrocatechol
IL-8 ng/10^6 cells
DMSO
50 μM
125 μM
250 μM
Etoposide
+ LPS